

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 38/46, 38/54, C07H 21/04, C12N 1/16, 1/20, 9/78, 15/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/33519</b> <b>(43) International Publication Date:</b> 6 August 1998 (06.08.98)
<b>(21) International Application Number:</b> PCT/US98/01635 <b>(22) International Filing Date:</b> 27 January 1998 (27.01.98) <b>(30) Priority Data:</b> 08/792,283 31 January 1997 (31.01.97) US <b>(71) Applicant:</b> ENZON, INC. [US/US]; 20 Kingsbridge road, Piscataway, NJ 08854 (US). <b>(72) Inventors:</b> FILPULA, David, Ray; 12 Carlton Club Drive, Piscataway, NJ 08854 (US). WANG, Maoling; 54 Rice Run, East Brunswick, NJ 08816 (US). <b>(74) Agents:</b> MERCANTI, Michael, N. et al.; Roberts & Mercanti, LLP, P.O. Box 484, Princeton, NJ 08542-0484 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ARGININE DEIMINASE DERIVED FROM <i>MYCOPLASMA ARTHRITIDIS</i> AND POLYMER CONJUGATES CONTAINING THE SAME  <b>(57) Abstract</b>  A purified arginine deiminase (ADI) obtained from <i>Mycoplasma arthritidis</i> having the amino acid sequence of SEQ ID NO:2 as well as an isolated nucleic acid molecule containing a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO:1 are disclosed. Other aspects of the invention include an expression vector, a cloned gene for expressing the <i>Mycoplasma arthritidis</i> derived ADI, (recombinant) host cells useful in expressing the ADI of the present invention and substantially non-antigenic polymer conjugates containing the ADI of the present invention as well as methods of treating arginine deiminase susceptible conditions in mammals. The arginine deiminase-polymer conjugates have high levels of retained enzyme activity and relatively long circulating lives.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**ARGININE DEIMINASE DERIVED FROM *MYCOPLASMA ARTHRITIDIS***  
**AND POLYMER CONJUGATES CONTAINING THE SAME**

**BACKGROUND OF THE INVENTION**

5       The present invention is directed to a novel arginine deiminase and long-acting arginine deiminase-containing compositions. In particular the invention is directed to substantially non-antigenic polymer conjugates containing arginine deiminase derived from *Mycoplasma arthritidis* which demonstrate high levels of retained enzyme activity.

10       Conjugating biologically-active proteins or enzymes to polymers has been suggested to improve one or more of the properties of circulating life, water solubility or antigenicity *in vivo*. For example, some of the initial concepts of coupling peptides or polypeptides to polyethylene glycol (PEG) and similar water-soluble polymers are disclosed in U.S. Patent No. 4,179,337, the disclosure of which is incorporated herein by reference. Conjugates are formed by reacting a  
15       biologically active material with a several fold molar excess of a polymer which has been modified to contain a terminal linking group. Insulin and hemoglobin were among the first therapeutic agents conjugated. These relatively large polypeptides contain several free  $\epsilon$ -amino attachment sites. Several polymers could be attached without significant loss of biologic activity.

20       The conjugation process, however, is not without complications. Excessive polymer conjugation or reactions using molar excesses of polymers beyond certain ratios can result in the formation of inactive conjugates or conjugates having insufficient activity. Problems often result when the active site (i.e. where groups associated with bioactivity are found) on the protein or enzyme becomes blocked as  
25       a result of the covalent polymer attachment. This problem can be difficult to avoid because the polymer and protein or enzyme are typically joined in solution-based reactions. Pre-blocking the active sites with reversible materials such as pyridoxal phosphate has been suggested, but the results have been inconsistent. The problems are particularly acute with relatively lower molecular weight proteins and peptides.  
30       These bioactive materials often have few attachment sites not associated with

bioactivity.

Arginine deiminase (ADI) is one type of enzyme which could benefit from improved polymer conjugation techniques. ADI is an enzyme that hydrolyzes arginine and the depletion of arginine has been postulated to have a killing effect on certain tumors. In particular, the enzyme hydrolyzes the guanidino group of L-arginine into L-citrulline and ammonia. The ADI enzyme is also known as arginine dihydrolase, arginine desiminase or guanidinodesiminase. Although ADI has been obtained from other types of mycoplasma strains as well as other microorganism sources such as pseudomonas and streptococcus, there is variability in the enzyme obtained. In particular, each host strain produces an enzyme having a different number of lysines as well as variations in the active site to produce an enzyme having a different primary sequence, and number and location of lysines.

Several polymer-arginine deiminase conjugates have previously been suggested. See, for example, Jpn. J. Cancer Res. 84, 1195-1200, Nov. 1993 which describes *inter alia* arginine deiminase from *Mycoplasma arginini* conjugated with methoxy-polyethylene glycol-4,6-dichloro-1,3,5-triazine. The previous arginine deiminase-polymer conjugates prepared to date, however, have been deemed to be unacceptable. One of the chief drawbacks has been that the level of retained arginine deiminase activity provided by highly modified conjugates has been too low in growth inhibition studies. It has been postulated that certain lysine attachment points on the enzyme are intimately connected with the enzyme active site. Therefore, the highly modified conjugates which demonstrate high levels of retained activity were not possible at reasonable expenditures of time and resources.

The present invention addresses these shortcomings.

## **SUMMARY OF THE INVENTION**

It is an object of the invention to provide a novel purified arginine deiminase subunit (hereinafter ADI) obtained from *Mycoplasma arthritidis* having the amino acid sequence of SEQ ID NO:2. In this regard, the invention includes an isolated nucleic acid molecule containing a nucleotide sequence encoding ADI comprising

the amino acid sequence set forth in the Figures and SEQ ID NO:1 and SEQ ID NO:2 as well as nucleic acid molecules complementary thereto.

Other aspects of the invention include an expression vector containing a cloned gene for *Mycoplasma arthritidis* derived ADI, (recombinant) host cells  
5 useful in expressing the ADI of the present invention and substantially non-antigenic polymer conjugates containing the ADI of the present invention. Still further aspects of the invention include a process for preparing the purified ADI, a process for preparing the aforementioned arginine deiminase-containing substantially non-antigenic polymer-based compositions as well as methods of treating arginine  
10 deiminase susceptible conditions in mammals. In this aspect, the treatment methods include administering an effective amount of the compositions described herein, preferably as part of a polymer conjugate, to mammals in need of such therapy.

The substantially non-antigenic polymer is preferably a polyalkylene oxide such as a polyethylene glycol having a molecular weight of from about 600 to about  
15 60,000 and preferably having a molecular weight of about 12,000. Other substantially non-antigenic polymers can also be used. The substantially non-antigenic polymer is preferably covalently conjugated to the ADI via a urethane or similarly hydrolysis resistant linkage in one preferred embodiment. The arginine deiminase-polymer conjugate containing compositions can be included as part of a  
20 pharmaceutically-acceptable solution.

The ADI obtained from *M. arthritidis* in accordance with the present invention differs from that previously reported in J. Biol. Chem. Vol. 253 No. 17 pp 6016-6020 (1978) and J. Biol. Chem. Vol. 252 No. 8 pp 2615-2620 (1977). These  
25 references described ADI enzymes designated as Type I, II and III enzymes, which were reported by the authors to be interconvertible and related proteins. Although the ADI enzymes reported in J. Biol. Chem. and the ADI of the invention were obtained from the same American Type Culture Collection ("ATCC") 14152 strain, the ADI of the invention is completely distinguishable from the previously reported Type I, II and III enzymes by a number of fundamental properties. Firstly, the ADI

described herein has only two cysteines per subunit sequence. The previously reported protein included eighteen cysteines per subunit. Secondly, the ADI of the present invention has a pI of about 5.25 whereas the previously reported ADI obtained from *M. arthritidis* had a pI of 7.0. In addition, the previous work reported above also predicted that the ADI obtained from *M. arthritidis* would have fewer lysines per subunit than the ADI from *M. arginini*. The present inventors, however, have surprisingly discovered that the opposite was the case. A still further point of difference is the fact that the N-terminal residue of the ADI of the present invention is serine(following post-translational removal of methionine), while the previously reported *M. arthritidis* ADI reported alanine as the N-terminal residue. These differences are dramatic and suggest that there is heterogeneity in the ADI obtained from obtained from *M. arthritidis*. A still further possibility is that there is more than one gene for ADI activity associated with *M. arthritidis*.

The arginine deiminase-polymer conjugates of the present invention also afford advantages over those of the prior art. For example, the ADI of the present invention includes several more modifiable lysine positions than prior art ADI, without resorting to the preparation of mutant lysine variants. This allows for substantially more polymer strands to be covalently attached to alternate surface locations without losing tumor cell growth inhibition activity. In addition, the thus formed conjugates have a substantially longer in vivo circulating life than conjugates having similar levels of retained activity prepared according to the prior art.

The term "arginine deiminase susceptible condition" shall be understood to include all disease states, such as tumor growths, cancers, or related conditions, which benefit therapeutically from exogenous arginine deiminase administration.

Details concerning such conditions are provided below in Section 4.

For a better understanding of the present invention, reference is made to the following description and its scope will be pointed out in the appended claims.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 illustrates the complete DNA sequence encoding ADI cloned from

*M. arthritidis* ATCC 14152 (1230 bases). The underlined G in the 5-tryptophan codons were changed from A to G by site directed mutagenesis.

Figure 2 illustrates the translation product (410 amino acids) of the arginine deiminase subunit from *M. arthritidis* ATCC 14152.

Figure 3 illustrates the amino acid sequence alignment of various arginine deiminases; line a: *M. arthritidis* ATCC 14152; line b: *M. arginini* LBIF, see Ohno et al. Infection and Immunity 58: Nov. 1990, pp 3788-3795; line c: *M. hominis* PG21; line d: *M. orale* FERM BP-1970. *M. hominis* and *M. orale* obtained according to R. Harasawa et al. Microbiol. Immunol. 36, pp 661-665, 1992.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **1. ARGININE DEIMINASE**

Accordingly, the present invention includes a novel protein comprising SEQ ID NO:2 having arginine deiminase enzyme activity and a nucleic acid molecule encoding the same. Preferably, the ADI is expressed by a novel gene, comprising the nucleic acid sequence of SEQ ID NO:1, that is isolated from *M. arthritidis*. The present invention also includes methods of making and using the same. In order for the reader to better appreciate the description to follow, the following terms are explained.

The nucleotide sequence of SEQ ID NO:1 is presented in the form of a deoxyribonucleic acid or DNA sequence. However, the artisan will understand that the nucleotide sequence of SEQ ID NO:1 can also be prepared in the form of an RNA molecule, as necessary. Further, the nucleotides comprising the DNA or RNA molecule can also be in the form of nucleotide derivatives or analogs, such as, for example, those listed at 37 C.F.R. § 1.822(p)(1), the disclosure of which is incorporated by reference herein in its entirety. In addition, the invention also encompasses the complement of the nucleotide sequences according to the invention. The artisan will appreciate the fact that the scope of the invention also includes alternate codons which can code for the same amino acid due to the degenerate nature of the genetic code.

"Transfection" refers to the taking up of an expression vector by a host cell, whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan. For example, transfection is accomplished in the presence of an expression vector and high concentrations of  $\text{CaPO}_4$ , by electroporation, by use of a phage or viral expression vector for insertion into a host cell, by mechanical insertion of nucleic acid, and even by culturing the host cells in the presence of unpackaged nucleic acid fragments. Successful transfection is generally recognized when any indication of the operation of the vector of interest occurs within the host cell.

"Transformation" describes the introduction of a nucleic acid into an organism so that the nucleic acid is replicable, either as an extrachromosomal element or by integration in the host chromosome. Depending on the host cell used, transformation is accomplished using art known methods appropriate to particular host cells. The calcium treatment employing calcium chloride, as described by Cohen, S. N. Proc. Natl. Acad. Sci. (USA), 69: 2110 (1972) and Mandel et al., J. Mol. Biol. 53:154 (1970), is generally used for prokaryotes or other cells that are encapsulated within cellular walls (e.g., many bacterial and/or plant cells). For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham, F. and van der Eb, A., Virology, 52: 456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen, P., et al., J. Bact., 130: 946 (1977) and Hsiao, C. L., et al., Proc. Natl. Acad. Sci. (USA) 76: 3829 (1979). However, any other art-known methods for introducing nucleic acid, e.g., DNA, into cells, such as, for example, by nuclear injection or by protoplast fusion, may also be used.

As used herein, the term "complementary" with respect to a nucleic acid refers to the (using Watson-Crick base pairing) opposite strand produced when a first nucleic acid molecule is replicated using that molecule as a template, to form a



new, second nucleic acid strand that is complementary to the first. In one aspect of the invention, two nucleic acid molecules are considered to be complementary, each to the other, when they hybridize or bind together under stringent conditions.

5 The expression "hybridize under stringent conditions" to describe the hybridization of nucleic acid molecules encompassed within the scope of this invention refers to hybridizing under conditions of high hybridization specificity, e.g., low ionic strength and high temperature for washing. Such stringent conditions include, for example, hybridization with 0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSO<sub>4</sub> at 50°C., or alternatively, in the presence of denaturing  
10 agents such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate, at 42°C for hybridization. "Hybridize under low stringency" refers to hybridizing under conditions of reduced hybridization specificity. Such conditions include, simply by  
15 way of example, hybridizing at 42°C. in 20% formamide, 5 x SSC, 50 mM sodium phosphate pH 6.8, 0.1% sodium pyrophosphate, 5 x Denhardt's solution, and 50 µg/ml salmon sperm DNA, and washing with 2 x SSC, 0.1% SDS at 42°C.

Additionally, specific mutations can be introduced into the arginine deiminase gene of the present invention using "site-directed mutagenesis". This is a  
20 technique standard in the art, and is conducted, e.g., using a synthetic oligonucleotide primer complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Such mutations may include, for example, the deletion, insertion, or substitution of the codons expressing naturally occurring amino acids. Such mutations may confer  
25 altered protein characteristics, which may, for example, improve and/or alter the oxidative, thermal, and/or pH stability of the protein. Briefly, in this method, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are

plated on agar, permitting plaque formation from single cells that harbor the phage. Usually from about 50 up to about 90% of the new plaques will contain the phage having, as a single strand, the mutated form. The plaques are hybridized with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques that hybridize with the probe are then selected and cultured, and the DNA is recovered. Thus, the artisan will appreciate that the nucleotide sequence of SEQ ID NO:1 can be conveniently subjected to mutagenesis by art known techniques, e.g., by nucleotide substitution, to produce useful variant alleles. Simply by way of example, the nucleotide sequence of SEQ ID NO:1 has been variously prepared with substitutions providing a T at nucleotide 39, a C at nucleotide 104, an A at nucleotide 206, a G at nucleotide 337, an A at nucleotide 729, a C at nucleotide 830, a C at nucleotide 1023, an A at nucleotide 6, a T at nucleotide 15, a C at nucleotide 18 and/or combinations thereof. In particular, the specific substitution at 337 would change the threonine to alanine.

"Operably linked" refers to a juxtaposition of components, e.g., a regulatory region and an open reading frame, such that the normal function of the components can be performed. Thus, an open reading frame that is "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences and wherein the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then, for example, synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

"Control Sequences" refers to nucleic acid sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize, for example, such control sequences as promoters, polyadenylation signals, and enhancers, to name but a few.

"Expression system" or "expression vector" refers to nucleic acid sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. To effect transformation, the expression system may be included on a vector; however, the relevant nucleic acid molecule may then also be integrated into the host chromosome.

As used herein, "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in genomic content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The vectors disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms. In general, prokaryotes are preferred for the initial cloning of DNA sequences and construction of the vectors useful in the invention. For example, *E. coli* K12 strain MM 294 (ATCC No. 31,446) is particularly useful. Other microbial strains, simply by way of example, that may be used include *E. coli* strains such as *E. coli* B and *E. coli* X1776 (ATCC No. 31,537). Prokaryotes may also be used for expression. The aforementioned strains, as well as, e.g., *E. coli* strains W3110 (F-, lambda-, prototrophic, ATCC

No. 27,325), K5772 (ATCC No. 53,635), and SR101, bacilli such as *Bacillus subtilis*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various pseudomonas species, may be used.

5 In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977, Gene, 2: 95). pBR322 contains  
10 genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. Similarly, the pUC plasmids provide convenient cloning vectors with DNA molecules for selection and replication (Yanisch-Perron, et al., 1985, Gene 33:103-119, the disclosure of which is incorporated by reference herein in its entirety). The pBR322 plasmid, or other microbial plasmid or phage,  
15 must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

"Plasmids" are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from  
20 such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

Those promoters most commonly used in recombinant DNA construction include the beta -lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978 Nature, 375: 615; Itakura et al., 1977, Science, 198: 1056; Goeddel et al.,  
25 1979, Nature, 281: 544) and a tryptophan (trp) promoter system (Goeddel et al., 1980, Nucleic Acids Res., 8: 4057; EPO Appl. Publ. No. 0036,776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published,

enabling a skilled worker to ligate them functionally with art known vectors, e.g., plasmid vectors.

Simply by way of example, transcriptional regulation in *E. coli* may be achieved with any of the following inducible promoters: lac, trp, phoA, araBAD, T7, and derivatives of the lambda P<sub>L</sub> and P<sub>R</sub> promoters as well as others well known to the art (e.g., Makrides, 1996, Microbiol. Rev. 60:512-538, the disclosure of which is incorporated by reference herein in its entirety). Preferably, the promoter is the O<sub>L</sub>/P<sub>R</sub> hybrid promoter, described by Scandella et al., in co-owned U.S. Patent No. 5,162,216, the disclosure of which is incorporated by reference herein in its entirety, is employed.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example (Stinchcomb et al., 1979, Nature, 282: 39; Kingsman et al., 1979, Gene, 7: 141; Tschemper et al., 1980, Gene, 10: 157), is commonly used. This plasmid already contains the trp1 gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 (Jones, 1977, Genetics, 85: 12). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

The *Pichia pastoris* expression system has been shown to achieve high level production of several proteins (Cregg, J.M. et al., 1993, Bio/Technology 11: 905-910, the disclosure of which is incorporated by reference herein in its entirety) and may be employed to express ADI as a soluble protein in the cytoplasm of *Pichia pastoris*.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. 1980, Biol. Chem., 255: 2073) or other glycolytic enzymes (Hess et al., 1968, J. Adv. Enzyme Reg., 7: 149 ; Holland et al.,

1978, Biochemistry, 17: 4900), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and transcription termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Satisfactory amounts of protein are produced by cell cultures; however, refinements, using a secondary coding sequence, serve to enhance production levels even further. One secondary coding sequence comprises dihydrofolate reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate (MTX), thus permitting control of expression by control of the methotrexate concentration.

Although any suitable strain of *M. arthritidis* can be employed as a source of the novel gene according to the invention, preferably, the *M. arthritidis* strain that is employed is the strain deposited in the American Type Culture Collection ("ATCC") as accession number 14152.

A gene capable of expressing the novel arginine deiminase enzyme according to the invention is preferably isolated from the *M. arthritidis* genome by primer directed nucleic acid amplification and thereafter cloned into any suitable screening vector and expression system (Molecular Cloning: A Laboratory Manual, Second Edition, J. Sambrook, E.F. Fritsch and T Maniatis, Eds., Cold Spring Harbor Press, 1989). The artisan will appreciate that any appropriate art-known nucleic acid amplification method, utilizing suitable primers, can be employed. Preferably, the amplification method is the polymerase chain reaction, as described, simply by way of example, by Mullis, in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, the disclosures of which are incorporated herein by reference in their entireties.

The artisan will also appreciate that any plasmid, phage or cosmid expression vector, inserted into the respective host cells, may be employed to express, screen and identify an amplified nucleic acid fragment encoding the arginine deiminase of the invention. Preferably, an *Escherichia coli* expression system is employed. More preferably a GX6712/pGX9401 *Escherichia coli* expression system is employed.

The primers used for amplification of the ADI gene according to the examples provided hereinbelow were oligonucleotides having the sequences of SEQ ID NOs 3 and 4, respectively. These primers successfully amplified the entire gene. As indicated in the Examples below, the primer of SEQ ID NO:3 differed from the corresponding N-terminal encoding region of the isolated gene (SEQ ID NO:1) by three base substitutions which did not alter the encoded peptide sequence. The three base substitutions, the numbering of which is based on the numbering of SEQ ID NO:1, are as follows: at base 6 A is substituted for T; at base 15 T is substituted for C; and at base 18 C is substituted for T.

Thus, the successful amplification of the entire gene having a sequence comprising SEQ ID NO:1 was surprising, since, as a result of the aforementioned mismatches, relative to the naturally occurring N terminal sequence of the isolated

ADI gene, the SEQ ID NO:3 primer annealed poorly to the target fragment, thus providing an explanation for the very weak PCR signal that resulted from amplification using the primer of SEQ ID NO:3.

5 The artisan will also appreciate that a primer based on the exact naturally occurring N-terminal sequence of SEQ ID NO:1 will also readily serve, with even better efficacy, at amplifying the ADI gene according to the invention.

Clones provided according to the examples hereinbelow include plasmid pEN232 comprising the ADI gene in expression vector pGX 9401. The initial two PCR isolates of the *M. arthritidis* ADI gene were also cloned into plasmid pBluescript II SK(-) (Stratagene Cloning Systems, La Jolla, CA) and designated pEN241 and pEN242. The N-terminal fragment cloning of an ADI gene segment into pBluescript II SK(-), described in Example 1B, included two independently analyzed clones designated pEN245 and pEN246. Independent transformations of *E. coli* DH5-alpha (GIBCO BRL, Gaithersburg, MD) with plasmids pEN241, pEN242, pEN245 and pEN246 produced *E. coli* clones designated EN243, EN244, EN247 and EN248, respectively.

20 Once a transfected or transformed host cell is obtained, a nucleic acid molecule that includes a sequence according to SEQ ID NO:1 is readily produced by culturing the host cell, and extracting and isolating the nucleic acid as desired, by methods well known to the art. Depending on the degree of purity desired, the extracted nucleic acid may be isolated, or where desired, substantially isolated by art known methods, to be free or substantially free of contaminating host cell proteins and nucleic acids. Similarly, host cells expressing the arginine deiminase protein encoded by the expression vectors according to the invention are cultured by methods suitable for the selected host cell.

25 For example, host cells are cultured until desired cell densities are achieved, and then the cells are separated from the growth medium and the protein is extracted and thereafter renatured according to art-known methods. In particular, the cells are separated from the culture medium to form a cell paste. The cell paste



is then re-suspended and then disrupted by standard methods, e.g., mechanical, ultrasonic and/or chemical disruption. Preferably, the cells are disrupted by processing in a Microfluidizer (Microfluidics Corp. Newton MA) followed by washing with a suitable surfactant, such as, for example, Triton X-100.

5           The resulting homogenate is denatured with guanidine HCl, 6 M and then diluted into refolding buffer (e.g., 10 mM  $K_2PO_4$ , pH 7.0), particulates removed, e.g., by centrifugation, followed by purification of the supernatant by standard methods, e.g., by Q Sepharose column chromatography, to provide substantially purified arginine deiminase, e.g., with a purity of about 60% and having a specific  
10           activity ranging from about 3 to about 25 IU/mg, or more, and preferably from about 5 to about 20 IU/mg. Additional concentration of the ADI can be achieved using a Centriprep-10, Amicon, Inc. Beverly, MA. Other similarly operating columns can also be used if desired.

## 2. NON-ANTIGENIC POLYMERS

15           In order to form the polymer - arginine deiminase conjugates of the present invention, polymers such as poly(alkylene oxides) (PAO's) are converted into activated forms, as such term is known to those of ordinary skill in the art. Thus, one or both of the terminal polymer hydroxyl end-groups, (i.e. the alpha and omega terminal hydroxyl groups) are converted into reactive functional groups which  
20           allows covalent conjugation. This process is frequently referred to as "activation" and the product is called an "activated poly(alkylene oxide)". Polymers containing both alpha and omega linking groups are referred to as bis-activated polyalkylene oxides. Other substantially non-antigenic polymers are similarly "activated" or functionalized. Among the substantially non-antigenic polymers, mono-activated  
25           polyalkylene oxides (PAO's), such as monomethoxy-polyethylene glycols are preferred. In alternative embodiments, homobifunctional bis-activated polymers such as bis-succinimidyl carbonate activated PEG are preferred.

          The activated polymers are thus suitable for reacting with arginine deiminase and forming ADI-polymer conjugates wherein attachment preferably occurs at

either the amino terminal  $\alpha$ -amino group or  $\epsilon$ -amino groups of lysines found on the ADI.

In one preferred aspect of the invention, carbamate (urethane) linkages are formed using the ADI  $\epsilon$  amino groups and the activated polyalkylene oxides. Preferably, the carbamate linkage is formed as described in commonly owned U.S. Patent No. 5,122,614, the disclosure of which is hereby incorporated by reference. This patent discloses the formation of mono- and bis- N-succinimidyl carbonate derivatives of polyalkylene oxides (SC-PEG). Alternatives include para-nitrophenyl carbonate and carbonyl imidazole activated polymers.

In another aspect of the invention, polymers activated with amide-forming linkers such as cyclic imide thione-activated polyalkylene oxides, succinimidyl esters or the like are used to effect the linkage between the arginine deiminase and polymer terminal groups, see for example, U.S. Patent No. 5,349,001 to Greenwald, et al., the disclosure of which is incorporated herein by reference. Still other aspects of the invention include using other activated polymers to form covalent linkages of the polymer with the arginine deiminase via  $\epsilon$  amino or other groups. For example, isocyanate or isothiocyanate forms of terminally activated polymers can be used to form urea or thiourea-based linkages with the lysine amino groups. PEG-dialdehyde can also be reacted with the arginine deiminase followed by reduction with  $\text{NaCNBH}_3$  to form a secondary amine linkage.

Suitable polymers will vary substantially by weight, however polymers having molecular weights ranging from about 200 to about 60,000 are usually selected for the purposes of the present invention. Molecular weights of from about 1,000 to about 40,000 are preferred and 2,000 to about 20,000 are particularly preferred.

The polymeric substances included are also preferably water-soluble at room temperature. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof,

provided that the water solubility of the block copolymers is maintained.

As an alternative to PAO-based polymers, effectively non-antigenic materials such as dextran, polyvinyl pyrrolidones, polyacrylamides, polyvinyl alcohols, carbohydrate-based polymers and the like can be used. Indeed, the activation of alpha and omega terminal groups of these polymeric substances can be effected in fashions similar to that used to convert polyalkylene oxides and thus will be apparent to those of ordinary skill. Those of ordinary skill in the art will realize that the foregoing list is merely illustrative and that all polymer materials having the qualities described herein are contemplated. For purposes of the present invention, "effectively non-antigenic" means all materials understood in the art as being nontoxic and not eliciting an appreciable immunogenic response in mammals.

### 3. REACTION CONDITIONS

Conjugation reactions, sometimes referred to as pegylation reactions, are generally carried out in solution with from about an equimolar to about a several fold molar excess of activated polymer. Preferably, the molar excess of activated polymer is about 50-fold, or greater. One way to maintain the arginine deiminase bioactivity is to substantially avoid including those arginine deiminase lysines associated with the active site in the polymer coupling process. Given the usually non-specific nature of the coupling reaction, this theoretical step is often difficult to achieve in practice. The process of the present invention, however, provides arginine deiminase conjugates having high levels of retained activity by using arginine deiminase obtained from *M. arthritidis*, which has a substantial increase in the number of available lysines for polymer attachment, and avoiding the use of an excessively high molar excess, e.g., more than about 100 fold, of activated polymer during the conjugation reactions.

Thus, the conjugation conditions include reacting arginine deiminase obtained from *M. arthritidis* with a suitably activated substantially non-antigenic polymer such as SC-PEG in a suitable buffer solution in a ratio of activated polymer to arginine deiminase of from about 50 to about 100 fold.

The conjugation reaction is carried out under relatively mild conditions to avoid inactivating the arginine deiminase. Mild conditions include maintaining the pH of the reaction solution in the range of 6-8 and the reaction temperatures within the range of from about 0 - 30° C and preferably at about 4°C for about one hour.

5 Suitable buffers include buffer solutions able to maintain the preferred pH range of 6-8 without interfering with the conjugation reaction. A non-limiting list of suitable buffers includes, e.g., phosphate buffer, citrate buffer, acetate buffer.

Although the reaction conditions described herein may result in some unmodified arginine deiminase, the unmodified arginine deiminase can be readily  
10 recovered and recycled into future batches for additional conjugation reactions.

The conjugation reactions of the present invention initially provide a reaction mixture or pool containing arginine deiminase conjugates having from about 16 to about 22 strands of polymer per subunit of enzyme (31 total lysines), unreacted arginine deiminase, if any, and unreacted polymer. After the unreacted  
15 species have been removed, compositions containing the arginine deiminase-polymer conjugates are recovered. These compositions have at least about 20% of the biological activity associated with the native or starting arginine deiminase as measured using an assay such as that described below in Example 3. In preferred aspects of the invention, however, the conjugates have at least about 30% of the  
20 biological activity associated with starting arginine deiminase and most preferably, the conjugates have at least about 90% of the biological activity associated with starting arginine deiminase.

A representative conjugation reaction is set forth below:

25 An about 50 fold molar excess of activated polymer is dissolved in Water For Injection (pH approximately 6.0) and then added to an *M. arthritidis* arginine deiminase solution adjusted to about pH 8.0 with a suitable buffer such as a phosphate or borate buffer. The reaction is allowed to incubate at about 4°C, at about pH 8.0, for a suitable time, such as about 1 hour, with continuous gentle mixing. Thereafter, the conjugation reaction is stopped, for example with a several-

fold molar excess of arginine or glycine. The unmodified arginine deiminase present in the reaction pool, if any, after the conjugation reaction has been quenched, can be recovered for recycling into future reactions using ion exchange or size exclusion chromatography or similar separation techniques. Preferably, solutions containing the conjugates of the present invention contain less than about 5% unmodified arginine deiminase.

If desired, the arginine deiminase-polymer conjugates are isolated from the reaction mixture to remove high molecular weight species, and unmodified arginine deiminase. The separation process is commenced by placing the mixed species in a buffer solution containing from about 1-10 mg/ml of the arginine deiminase-polymer conjugates. Suitable solutions have a pH of from about 6.0 to about 9.0 and preferably from about 7.5 to about 8.5. The solutions preferably contain one or more buffer salts selected from KCl, NaCl,  $K_2HPO_4$ ,  $KH_2PO_4$ ,  $Na_2HPO_4$ ,  $NaH_2PO_4$ ,  $NaHCO_3$ ,  $NaBO_3$ , and NaOH. Sodium phosphate buffers are preferred.

Depending upon the reaction buffer, the arginine deiminase polymer conjugate solution may first have to undergo buffer exchange/ultrafiltration to remove any unreacted polymer. For example, the PAO-Arginine deiminase conjugate solution can be ultra-filtered across a low molecular weight cut-off (10,000 to 30,000 Dalton) membrane to remove most unwanted materials such as unreacted polymer, surfactants, if present, or the like.

Fractionation of the ADI-polymer conjugates, if desired, can also be carried out using an anion exchange chromatography medium. Such media are capable of selectively binding PAO-arginine deiminase conjugates via differences in charge which vary in a somewhat predictable fashion. For example, the surface charges of ADI is determined by the number of available charged amino acids on the surface of the protein. Of these charged amino acids, lysine residues serve as the point of potential attachment of polyalkylene oxide conjugates. Therefore, arginine deiminase conjugates will have a different charge from the other species to allow selective isolation. The use of strongly polar anion exchange resins such as

quaternary amine anion exchange resins are especially preferred for the method of the present invention. Included among the commercially available quaternary anion exchange resins suitable for use with the present invention are Q-HD, QA TRISACRYL® and QMA-SPHEROSIL®, quaternary amine resins coated onto a polymer matrix, manufactured by IBF of Garenne, France, for Sepracor of Marlborough, Massachusetts; TMAE650M®, a tetramethylamino ethyl resin coated onto a polymer matrix, manufactured by EM-Separators of Gibbstown, New Jersey; QAE550C®, and SUPERQC®, each a quaternary amine resin coated onto a polymer matrix and manufactured by TosoHaas of Montgomeryville, PA. QMA Accell, manufactured by Millipore of Millford, MA and PEI resins manufactured by JT Baker of Phillipsburg, NJ, may also be used. Other suitable anion exchange resins e.g. DEAE resins can also be used.

For example, the anion exchange resin is preferably packed in a column and equilibrated by conventional means. A buffer having the same pH and osmolality as the polymer conjugated arginine deiminase solution is used. The elution buffer preferably contains one or more salts selected from KCl, NaCl,  $K_2HPO_4$ ,  $KH_2PO_4$ ,  $Na_2HPO_4$ ,  $NaH_2PO_4$ ,  $NaHCO_3$ ,  $NaBO_4$ , and  $(NH_4)_2CO_3$ . The conjugate-containing solution is then adsorbed onto the column with the high molecular weight species and unreacted polymer not being retained. At the completion of the loading, a gradient flow of an elution buffer with increasing salt concentrations is applied to the column to elute the desired fraction of polyalkylene oxide-conjugated arginine deiminase. The eluted pooled fractions are preferably limited to uniform mono- and bis-arginine deiminase polymer conjugates after the anion exchange separation step. Any unconjugated arginine deiminase species can then be back washed from the column by conventional techniques. If desired, the arginine deiminase species can also be separated via additional ion exchange chromatography or size exclusion chromatography. The temperature range for elution is between about 4°C and about 25°C. Preferably, elution is carried out at a temperature of from about 6°C to about 22°C. Fraction collection may be achieved through simple time elution

profiles.

#### 4. METHODS OF TREATMENT

Another aspect of the present invention provides methods of treatment for various medical conditions in mammals. The methods include administering an effective amount of arginine deiminase-polymer conjugates which have been prepared as described herein to a mammal in need of such treatment. The conjugates are useful for, among other things, treating arginine deiminase-susceptible conditions or conditions which would respond positively or favorably as these terms are known in the medical arts to arginine deiminase-based therapy.

While not wishing to be bound by any hypothesis or theory, the artisan will appreciate that arginine is a naturally occurring amino acid that is considered to be a non-essential nutrient in the human diet. A mammal having a condition, disease or disorder which will benefit from a decrease in the amount or concentration of arginine in the tissue, cells or circulating fluids of a mammal, will benefit from such treatment with ADI-polymer conjugates according to the invention. Arginine deiminase catalyses the direct conversion of L-arginine and H<sub>2</sub>O to l-citrulline and NH<sub>3</sub>.

Thus, without limitation, the arginine deiminase conjugates can be used to treat conditions, including, carcinomas deficient in the enzyme argininosuccinate synthetase, e.g., melanoma (Sugimura et al., 1992, Melanoma Res. 2:191-196) and nitric oxide (NO) related conditions, e.g., conditions that may be treated or ameliorated by modulation of nitric oxide synthase. In particular, it has been shown that cellular NO production is absolutely dependent on availability of arginine. (Nagasaki et al, 1996, J. Biol. Chem. 271:2658-2662; Xia et al., 1996, Proc. Natl. Acad. Sci. USA 93:6770-6774). ADI also has certain dietary applications, e.g., in modulating the negative effects of low protein diets (Narita et al., 1995, Proc. Natl. Acad. Sci. USA 92:4552-4556).

The amount of the arginine deiminase-polymer conjugate administered to treat the conditions described above is based on the arginine deiminase activity of the

polymeric conjugate. It is an amount that is sufficient to significantly effect a positive clinical response. The maximal dose for mammals including humans is the highest dose that does not cause clinically-important side effects. For purposes of the present invention, such clinically important side effects are those which would require cessation of therapy such as, for example, hypersensitivity reactions and/or other immunogenic reactions.

Naturally, the dosages of the arginine deiminase-based compositions will vary somewhat depending upon the arginine deiminase moiety and polymer selected. In general, however, the conjugate is administered in amounts ranging from about 300 to about 3000 IU/m<sup>2</sup> of arginine deiminase per day, based on the mammal's condition. The range set forth above is illustrative and those skilled in the art will determine the optimal dosing of the conjugate selected based on clinical experience and the treatment indication.

The ADI-polymer conjugates of the present invention can be included in one or more suitable pharmaceutical compositions for administration to mammals. The pharmaceutical compositions may be in the form of a solution, suspension, tablet, capsule or the like, prepared according to methods well known in the art. It is also contemplated that administration of such compositions will be chiefly by the parenteral route although oral or inhalation routes may also be used depending upon the needs of the artisan.

## **EXAMPLES**

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

### **EXAMPLE 1**

#### **Expression of *M. arthritidis* ADI Gene in *E. coli***

##### **A. Isolation and Cloning of ADI Gene**

*M. arthritidis* strain 14152 was obtained from the American Type Culture



Collection. The arginine deiminase gene of *M. arthritidis* was amplified by a polymerase chain reaction (PCR) using the primer pair  
5' GGCAATCGATGT CTGTATTTGACAGTA-3' (SEQ ID NO:3) and 5'-  
TGAGGATCCTTACTACCACTTAACATCTTTACG-3' (SEQ ID NO:4) derived  
5 from the published sequence of *M. arginini* LBIF (Ohno, T. et al. 1990) "Cloning and Nucleotide Sequence of the Gene Encoding Arginine Deiminase of *Mycoplasma arginini*" Infect. Immun. 58: 3788-3795, the contents of which are incorporated herein by reference.

The PCR amplification was conducted by standard methods ( as reviewed by  
10 Saiki et al., 1989, PCR Technology, pages 7-16; Ed. Henry A. Erlich, Stockton Press) with the following parameters.

The reaction was conducted in a volume of 100 microliters, with a PCR  
buffer of 10 millimolar Tris-HCl, pH 8.3, 50 millimolar KCl, 2 millimolar MgCl<sub>2</sub>, and  
200  $\mu$ M of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and  
15 2.5 units of Taq DNA polymerase (from Perkin Elmer). Thirty cycles of  
amplification were carried out in a Perkin Elmer PCR system 9600 thermal cycler,  
set to denature at 94 degrees C. for 60 seconds, anneal at 37°C for 180 seconds and  
extend at 72 degrees C for 120 seconds.

After PCR amplification, two rather faint bands representing amplified  
20 fragments were observed on agarose gel analysis at 1.4 kb and 1.2 kb. Samples of  
each fragment, respectively, were excised from the gel, purified and cloned as a  
*Cla*I-*Bam*HI fragment directly into expression plasmid pGX9401 in the manner  
disclosed by Filpula et al. in "Engineering of Immunoglobulin Fc and Single Chain  
Fv Proteins in *Escherichia coli*" in Antibody Expression and Engineering (H. Y.  
25 Wang and T. Imanaka, eds.) American Chemical Society, pp 70-85. the contents of  
which are incorporated herein by reference. Both fragments were sequenced, and the  
1.2 kb fragment was confirmed as encoding ADI by its partial homology to  
previously known genes encoding enzymes with ADI enzyme activity.

The five TGA codons in the isolated ADI gene which encode tryptophan in

*Mycoplasma* were changed to TGG codons by oligonucleotide-directed mutagenesis according to the method of Sayers et al. Biotechniques 13: 592-596 (1992), the disclosure of which is incorporated herein by reference, prior to gene expression in *E. coli*. The GX6712/pGX9401 *E. coli* expression system used was the same as that described in the aforementioned Filpula et al. reference. Recombinant ADI was expressed in inclusion bodies at levels of 10% of the total cell protein.

#### B. Confirmation of N-Terminal Sequence of ADI Gene

In order to confirm the N-terminal region of the *M. arthritis* gene corresponding to PCR primer SEQ ID NO:3, an independent PCR amplification of the N-terminal region was conducted using the established DNA sequence data from the first PCR. The technique employed was that of "inverse PCR" as described by H. Ochman et al., 1990, (PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS, Academic Press, Inc., Eds., M.A. Innis, D.H. Gelfand et al.).

The inverse PCR was conducted using with PCR primers 5'-CTAAAACGGTTTCTAGTTCACC -3' (SEQ ID NO:5) and 5'- AGCGTGGAATTAATGTTGTTG -3' (SEQ ID NO:6). Two micrograms of genomic *M. arthritis* DNA was digested with restriction endonuclease Sau 3A, then fragments were circularized by treatment with T4 DNA ligase. This DNA preparation was then subjected to PCR amplification using SEQ ID NOs 5 and 6. The amplified DNA was cloned and analyzed by DNA sequencing. DNA sequence analysis confirmed the assignment of serine as the amino acid following the initiation methionine (which is predicted to be post-translationally removed). Three silent base changes were also noted: Base 6 is A rather than T; Base 15 is T rather than C; Base 18 is C rather than T. In addition, Base 39 is T rather than C in this cloned sequence. None of these changes alters the translated protein sequence.

The three base differences between the N-terminal coding region of the confirmed genomic sequence and the SEQ ID NO:3 primer employed to isolate the gene are believed to account for the rather faint bands, as discussed above, that were produced when the PCR product was analyzed by gel electrophoresis. The three

base differences are believed to have resulted in a poor annealing between the primer and the N-terminal coding region, resulting in a weak PCR signal observed on gel electrophoresis. Thus, given this difference and the weak PCR signal, the successful amplification of the entire ADI gene by PCR required careful selection of PCR conditions and therefore successful isolation of the gene represented by SEQ ID NO:1 was unexpected.

## **EXAMPLE 2**

### **Renaturation and purification of Recombinant ADI**

In this example, the ADI protein obtained as result of Example 1 is renatured according to the techniques reported by Misawa et al. , with minor modifications (Misawa et al., 1994 "High-Level Expression of *Mycoplasma* arginine deiminase in *Escherichia coli* and Its Efficient Renaturation As An Antitumor Enzyme" in J. Biotechnol. 36: 145-155, the contents of which are incorporated herein by reference in its entirety).

To begin, 100 grams of cell paste is resuspended in 800 ml of 10 mM  $K_2PO_4$ , pH 7.0, 1 mM EDTA (buffer 1) and the cells are disrupted by two passes in a Microfluidizer (Microfluidics Corp. Newton MA). Triton X-100 is added to achieve a final concentration of 4% (v/v). The homogenate is stirred for 30 minutes at 4 degrees C. and is then centrifuged for 30 minutes at 13,000 g. The pellet is collected and re-suspended in one liter of buffer 1 containing 0.5% Triton X-100. The solution is dialfiltered against 5 volumes of denaturation buffer (50 mM Tris HCl, pH 8.5, 10 mM DTT) using hollow fiber cartridges with 100 kD retention rating (Microgon Inc. Laguna Hills, CA). Guanidine HCl is added to achieve a final concentration of 6 M and the solution is stirred for 15 minutes at 4 degrees C. The solution is then diluted 100-fold into refolding buffer (10 mM  $K_2PO_4$ , pH 7.0) and stirred for 48 hours at 15 degrees C. Particulates are removed by centrifugation at 13,000 g. The resultant supernatant is concentrated on a Q Sepharose Fast Flow (Pharmacia, Inc. Piscataway, NJ) column pre-equilibrated in refolding buffer. ADI is eluted using refolding buffer containing 0.2 M NaCl. The purification procedure

yields ADI protein which is > 95% pure as estimated SDS-PAGE analysis. About eight grams of pure renatured ADI protein are produced from 1 kilogram of cell paste, which corresponds to a yield of 200 milligrams of ADI per liter of fermentation.

### EXAMPLE 3

#### Arginine Deiminase Assay

ADI activity was determined by a minor modification of the method described by Oginsky et al. in "Isolation and Determination of Arginine and Citrulline" Methods Enzymology 3: 639-643 (1957), the disclosure of which is incorporated herein by reference. Ten microliter samples in 0.1 M  $\text{Na}_2\text{PO}_4$ , pH 7.0 (BUN assay buffer) were placed in a 96 well microtiter plate, 40 microliters of 0.5 M arginine in BUN assay buffer was added and the plate was covered and incubated at 37° C for 15 minutes. 200 microliters of complete BUN reagent (Sigma Diagnostics) was added and the covered plate was incubated for 10 minutes at 100° C. The plate was cooled to 22° C and analyzed at 490 nm by a microtiter plate reader (Molecular Devices, Inc.). One IU is the amount of enzyme which converts 1 micromole of L-arginine to L-citrulline per minute. Protein concentrations were determined using Pierce Coomassie Plus Protein Assay Reagent (Pierce Co., Rockford, IL) with bovine serum albumin as standard. The specific enzyme activity of the purified ADI preparations was determined to be about 3 to about 30 IU/mg.

### EXAMPLE 4

In this example, succinimidyl carbonate-activated monomethoxy-polyethylene glycol, molecular weight 12,000, was used to modify the arginine deiminase obtained as a result of Example 2. The succinimidyl carbonate activated mPEG was prepared in accordance with the method of the aforementioned U.S. Patent No. 5,122,614.

A solution of arginine deiminase (0.910 mg in a volume of 1 ml) was adjusted to pH 8.0 with 100 mM borate buffer. A 50-fold molar excess of the

activated PEG was dissolved in Water For Injection and then added to the arginine deiminase. The reaction was incubated at 4°C for about 1 hour with continuous gentle mixing. After 1 hour, the reaction was stopped with an excess of arginine. The PEG-ADI conjugates were diafiltered through a Centriprep 30 with 15 volumes of 0.1 molar sodium phosphate, pH 7.0, which was monitored at 220 nm for the presence of the polymer until < 0.05. The reaction products were analyzed as described in Example 3, *supra*, and found to have about 40 % retained ADI activity.

#### EXAMPLE 5

The process of Example 4 is repeated except that molecular weight 12,000 mPEG activated with an N-acyl thiazolidine is used.

#### EXAMPLE 6

The process of Example 4 is repeated except that succinimidyl carbonate-activated monomethoxy-polyethylene glycol, molecular weight 5,000, is used.

#### EXAMPLE 7

##### PEG<sub>12,000</sub>-ADI INHIBITION OF SK-MEL-2 CELL GROWTH

In this example, the PEG-ADI prepared in accordance with Example 4 was compared to PEG ADI conjugates made using ADI obtained from *M. arginini* in a cell growth inhibition assay. The *M. arginini* ADI was obtained in a similar fashion as that used to obtain the *M. arthritidis* ADI except that the *M. arginini* ADI was purified to a higher extent than the *M. arthritidis* ADI. In particular, the *M. arthritidis* ADI was extracted and refolded to about 60% purity but not processed through anion exchange chromatography. Processing through the anion exchange chromatography is expected to yield greater than 90% purity of the *M. arthritidis* ADI enzyme. The PEG- conjugation technique used to make the *M. arginini*-derived ADI conjugates was the same as that used in Example 4. In both cases, PEG MW 12,000 was used to make the conjugates.

Melanoma cells were growth in Minimum Essential Medium (Eagle) with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and Earle's salts; fetal bovine

serum 10%. Following trypsinization, viable cells were counted by trypan blue exclusion. Cells ( $10^4$ ) were added to each well in a total of 100 microliters in 96 well micro-titer plates. PEG<sub>12,000</sub> - ADI conjugates were diluted by 2-fold serial dilution in complete media (0.1 ml) and added to each well. Plates were incubated at 37° C in 5% CO<sub>2</sub> incubator. Cell growth at day 3 was measured by adding 1/10th volume of "Alamar Blue" dye (Alamar Biosciences, Inc. Sacramento, CA). After five hours of incubation, the plates were read with a Molecular Device plate reader at 570-630 nm.

The *M. arginini* derived PEG-ADI conjugates were found to have an IC<sub>50</sub> of 0.00015 IU/ml while the *M. arthritidis* - derived PEG-ADI had an IC<sub>50</sub> of 0.00010 IU/ml. Thus, when normalized in units of IU/ml, the PEG-ADI derived from *M. arthritidis* is seen to have 150% of the potency of the PEG-ADI derived from *M. arginini*.

#### EXAMPLE 8

##### SDS PAGE Analysis

An SDS-PAGE analysis was carried out to compare PEG<sub>12,000</sub>-ADI conjugates prepared either with *M. arginini*-derived ADI or, in accordance with the present invention, with *M. arthritidis*-derived ADI. The results indicate that the *M. arthritidis* derived conjugates had a more uniform distribution of size and had a higher average molecular weight (and therefore had more PEG molecules attached per ADI subunit). While Applicants are not bound by theory, it is believed that the *M. arthritidis* derived ADI included a greater number of lysines upon which the PEG could covalently attach, and, perhaps more importantly, there are a sufficient number of lysines on this specific ADI which are not associated with the active site.

#### EXAMPLE 9

##### Sequence Comparisons

In this example, the amino acid sequences and sequence alignment of various arginine deiminases were investigated. Turning to Figure 3, it is noted that line "a" represents the *M. arthritidis* ATCC 14152 ADI used in accordance with the present

invention. Line "b" represents *M. arginini* LBIF ADI. Line "c" represents *M. hominis* PG21 ADI and line "d" represents *M. orale* FERM BP-1970 ADI. The dashes indicate amino acids identical to those found in line "a". Dots indicate gaps. The percent of amino acid sequence identity to *M. arthritidis* ADI is:

5            *M. arginini*- 87%

*M. hominis*- 81%

*M. orale*-83%.

            This level of non-homology between the encoded amino acid sequences of the respective genes indicates significant differences between the encoded proteins of  
10            the respective *Mycoplasma* species. The sites of lysine substitutions are dispersed and extensive, indicating great diversity in potential polymer conjugation sites.

            While there have been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will realize that changes and modifications may be made thereto without departing from the spirit of  
15            the invention. It is intended to claim all such changes and modifications that fall within the true scope of the invention. Numerous references are cited in the specification, the disclosures of which are incorporated by reference in their entireties.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Enzon, Inc.  
20 Kingsbridge Road  
Piscataway, New Jersey 08854-3998
- (ii) TITLE OF INVENTION: Arginine Deiminase Derived From  
Mycoplasma and Polymer Conjugates Containing the Same
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: ROBERTS & MERCANTI
  - (B) STREET: 81 Tamarack Circle
  - (C) CITY: Skillman
  - (D) STATE: New Jersey
  - (E) COUNTRY: United States
  - (F) ZIP: 08558
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette 3.5 inch 1.4 Mb
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: Not yet available
  - (B) FILING DATE: 27-JAN-1998
  - (C) CLASSIFICATION: Not yet available
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/792,283
  - (B) FILING DATE: 31-JAN-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Mercanti, Michael N.
  - (B) REGISTRATION NUMBER: 33966
  - (C) REFERENCE/DOCKET NUMBER: 2131055PCT
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 609-921-3500
  - (B) TELEFAX: 609-921-9535

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1230 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: not relevant



(ii) MOLECULE TYPE: DNA (genomic)  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Mycoplasma arthritidis*  
 (B) INDIVIDUAL/ISOLATE: EN231  
 (C) CELL TYPE: unicellular organism

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG TCT GTA TTT GAC AGT AAA TTT AAG GGA ATT CAT GTC TAT TCA GAA	48
Met Ser Val Phe Asp Ser Lys Phe Lys Gly Ile His Val Tyr Ser Glu	
1 5 10 15	
ATT GGT GAA CTA GAA ACC GTT TTA GTT CAC GAA CCT GGT AAA GAA ATT	96
Ile Gly Glu Leu Glu Thr Val Leu Val His Glu Pro Gly Lys Glu Ile	
20 25 30	
GAT TAC ATT ACC CCA GCT CGT TTG GAT GAA TTA TTA TTC TCA GCT ATT	144
Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe Ser Ala Ile	
35 40 45	
CTA GAA AGC CAC GAT GCA AGA AAA GAA CAC AAA GAA TTC GTA GCA GAA	192
Leu Glu Ser His Asp Ala Arg Lys Glu His Lys Glu Phe Val Ala Glu	
50 55 60	
CTT AAA AAG CGT GGA ATT AAT GTT GTT GAA TTA GTA GAT CTA ATC GTA	240
Leu Lys Lys Arg Gly Ile Asn Val Val Glu Leu Val Asp Leu Ile Val	
65 70 75 80	
GAA ACC TAT GAT TTA GCA TCA AAA GAA GCT AAA GAA AAA CTT TTA GAA	288
Glu Thr Tyr Asp Leu Ala Ser Lys Glu Ala Lys Glu Lys Leu Leu Glu	
85 90 95	
GAA TTC CTA GAT GAT TCA GTA CCA GTT CTA TCA GAC GAA CAC CGT GCT	336
Glu Phe Leu Asp Asp Ser Val Pro Val Leu Ser Asp Glu His Arg Ala	
100 105 110	
ACT GTT AAG AAA TTC TTA CAA AGT CAA AAA TCA ACA AGA TCA TTA GTT	384
Thr Val Lys Lys Phe Leu Gln Ser Gln Lys Ser Thr Arg Ser Leu Val	
115 120 125	
GAA TAC ATG ATC GCA GGG ATC ACT AAA CAC GAT TTA AAA ATC GAA TCA	432
Glu Tyr Met Ile Ala Gly Ile Thr Lys His Asp Leu Lys Ile Glu Ser	
130 135 140	
GAT TTA GAA TTA ATC GTT GAC CCA ATG CCT AAC TTG TAC TTC ACT CGT	480
Asp Leu Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg	
145 150 155 160	
GAC CCA TTT GCA TCA GTA GGT AAT GGA GTT ACC ATC CAC TAC ATG CGT	528
Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Tyr Met Arg	
165 170 175	
TAC AAA GTA AGA CAA CGT GAA ACA TTA TTT AGC CGA TTT GTA TTT TCA	576
Tyr Lys Val Arg Gln Arg Glu Thr Leu Phe Ser Arg Phe Val Phe Ser	
180 185 190	

AAT CAC CCT AAA CTA GTT AAT ACC CCA TGG TAC TAC GAC CCT GCT GAA Asn His Pro Lys Leu Val Asn Thr Pro Trp Tyr Tyr Asp Pro Ala Glu 195 200 205	624
GGA TTA ACA ATC GAA GGT GGA GAC GTA TTT ATC TAC AAT AAC GAT ACT Gly Leu Thr Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr 210 215 220	672
TTA GTA GTT GGT GTT TCA GAA AGA ACT GAC TTA CAA ACT ATT ACT TTA Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Gln Thr Ile Thr Leu 225 230 235 240	720
TTA GCT AAG AAC ATT AAA GCA AAT AAA GAA TGT GAA TTC AAA CGT ATT Leu Ala Lys Asn Ile Lys Ala Asn Lys Glu Cys Glu Phe Lys Arg Ile 245 250 255	768
GTA GCA ATT AAT GTT CCT AAA TGG ACA AAC CTA ATG CAC TTA GAC ACA Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr 260 265 270	816
TGG TTA ACA ATG CTA GAC AAA GAT AAA TTC TTA TAC TCA CCT ATT GCA Trp Leu Thr Met Leu Asp Lys Asp Lys Phe Leu Tyr Ser Pro Ile Ala 275 280 285	864
AAT GAT GTG TTT AAA TTC TGG GAC TAC GAT TTA GTT AAT GGC GGA GAC Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Asp 290 295 300	912
GCT CCT CAA CCA GTT GAC AAT GGA TTA CCT CTA GAA GAC TTA TTG AAA Ala Pro Gln Pro Val Asp Asn Gly Leu Pro Leu Glu Asp Leu Leu Lys 305 310 315 320	960
TCA ATC ATT GGT AAG AAA CCT ACT CTA ATT CCT ATT GCT GGT GCT GGT Ser Ile Ile Gly Lys Lys Pro Thr Leu Ile Pro Ile Ala Gly Ala Gly 325 330 335	1008
GCT TCA CAA ATC GAT ATT GAA CGT GAA ACC CAC TTT GAC GGA ACA AAC Ala Ser Gln Ile Asp Ile Glu Arg Glu Thr His Phe Asp Gly Thr Asn 340 345 350	1056
TAC CTA GCT GTA GCT CCT GGA ATT GTT ATT GGT TAT GCA CGT AAC GAA Tyr Leu Ala Val Ala Pro Gly Ile Val Ile Gly Tyr Ala Arg Asn Glu 355 360 365	1104
AAA ACA AAT GCC GCT TTA GAA GCT GCA GGA ATT ACT GTT CTA CCA TTC Lys Thr Asn Ala Ala Leu Glu Ala Ala Gly Ile Thr Val Leu Pro Phe 370 375 380	1152
AGA GGA AAC CAA CTT TCA CTT GGA ATG GGA AAT GCT CGT TGC ATG TCA Arg Gly Asn Gln Leu Ser Leu Gly Met Gly Asn Ala Arg Cys Met Ser 385 390 395 400	1200
ATG CCT CTA TCA CGT AAA GAT GTT AAG TGG Met Pro Leu Ser Arg Lys Asp Val Lys Trp 405 410	1230

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 410 AMINO ACID RESIDUES

(B) TYPE: AMINO ACID

(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: PROTEIN

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Mycoplasma Arthritidis

(B) INDIVIDUAL/ISOLATE: EN231

(C) CELL TYPE: unicellular organism

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Ser Val Phe Asp Ser Lys Phe Lys Gly Ile His Val Tyr Ser Glu
 1             5             10             15

Ile Gly Glu Leu Glu Thr Val Leu Val His Glu Pro Gly Lys Glu Ile
      20             25             30

Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe Ser Ala Ile
      35             40             45

Leu Glu Ser His Asp Ala Arg Lys Glu His Lys Glu Phe Val Ala Glu
 50             55             60

Leu Lys Lys Arg Gly Ile Asn Val Val Glu Leu Val Asp Leu Ile Val
 65             70             75             80

Glu Thr Tyr Asp Leu Ala Ser Lys Glu Ala Lys Glu Lys Leu Leu Glu
      85             90             95

Glu Phe Leu Asp Asp Ser Val Pro Val Leu Ser Asp Glu His Arg Ala
      100            105            110

Thr Val Lys Lys Phe Leu Gln Ser Gln Lys Ser Thr Arg Ser Leu Val
      115            120            125

Glu Tyr Met Ile Ala Gly Ile Thr Lys His Asp Leu Lys Ile Glu Ser
      130            135            140

Asp Leu Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg
      145            150            155            160

Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Tyr Met Arg
      165            170            175

Tyr Lys Val Arg Gln Arg Glu Thr Leu Phe Ser Arg Phe Val Phe Ser
      180            185            190

Asn His Pro Lys Leu Val Asn Thr Pro Trp Tyr Tyr Asp Pro Ala Glu
      195            200            205

Gly Leu Thr Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr
      210            215            220

```

```

Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Gln Thr Ile Thr Leu
225                230                235                240

Leu Ala Lys Asn Ile Lys Ala Asn Lys Glu Cys Glu Phe Lys Arg Ile
                245                250                255

Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr
                260                265                270

Trp Leu Thr Met Leu Asp Lys Asp Lys Phe Leu Tyr Ser Pro Ile Ala
                275                280                285

Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Asp
                290                295                300

Ala Pro Gln Pro Val Asp Asn Gly Leu Pro Leu Glu Asp Leu Leu Lys
305                310                315                320

Ser Ile Ile Gly Lys Lys Pro Thr Leu Ile Pro Ile Ala Gly Ala Gly
                325                330                335

Ala Ser Gln Ile Asp Ile Glu Arg Glu Thr His Phe Asp Gly Thr Asn
                340                345                350

Tyr Leu Ala Val Ala Pro Gly Ile Val Ile Gly Tyr Ala Arg Asn Glu
                355                360                365

Lys Thr Asn Ala Ala Leu Glu Ala Ala Gly Ile Thr Val Leu Pro Phe
                370                375                380

Arg Gly Asn Gln Leu Ser Leu Gly Met Gly Asn Ala Arg Cys Met Ser
385                390                395                400

Met Pro Leu Ser Arg Lys Asp Val Lys Trp
                405                410

```

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

## (iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mycoplasma arthritidis
- (B) STRAIN: ATCC 14152
- (C) INDIVIDUAL ISOLATE: EN231
- (G) CELL TYPE: unicellular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCAATCGAT GTCTGTATTT GACAGTA

27

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mycoplasma arthritidis
- (B) STRAIN: ATCC 14152
- (C) INDIVIDUAL ISOLATE: EN231
- (G) CELL TYPE: unicellular organism

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAGGATCCT TACTACCACT TAACATCTTT ACG

33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mycoplasma arthritidis
- (B) STRAIN: ATCC 14152
- (C) INDIVIDUAL ISOLATE: EN231
- (G) CELL TYPE: unicellular organism

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAAAACGGT TTCTAGTTCA CC

22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: not relevant

- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "oligonucleotide"

- (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Mycoplasma arthritidis
  - (B) STRAIN: ATCC 14152
  - (C) INDIVIDUAL ISOLATE: EN231
  - (G) CELL TYPE: unicellular organism

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCGTGGAAT TAATGTTGTT G

21

- (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 410 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: unknown

- (ii) MOLECULE TYPE: protein

- (iii) HYPOTHETICAL: NO

- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Mycoplasma arginini
  - (B) STRAIN: LBIF
  - (G) CELL TYPE: unicellular organism

- (x) PUBLICATION INFORMATION:
  - (A) AUTHORS: Ohno, et al.,
  - (C) JOURNAL: Infection and Immunity
  - (D) VOLUME: 58
  - (F) PAGES: 3788-3795
  - (G) DATE: November-1990

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Ser	Val	Phe	Asp	Ser	Lys	Phe	Lys	Gly	Ile	His	Val	Tyr	Ser	Glu
1				5					10					15	
Ile	Gly	Glu	Leu	Glu	Ser	Val	Leu	Val	His	Glu	Pro	Gly	Arg	Glu	Ile
		20						25					30		
Asp	Tyr	Ile	Thr	Pro	Ala	Arg	Leu	Asp	Glu	Leu	Leu	Phe	Ser	Ala	Ile
		35					40					45			

Leu Glu Ser His Asp Ala Arg Lys Glu His Lys Ser Phe Val Ala Glu  
 50 55 60

Leu Lys Ala Asn Asp Ile Asn Val Val Glu Leu Ile Asp Leu Val Ala  
 65 70 75 80

Glu Thr Tyr Asp Leu Ala Ser Gln Glu Ala Lys Asp Lys Leu Ile Glu  
 85 90 95

Glu Phe Leu Asp Asp Ser Glu Pro Val Leu Ser Glu Glu His Lys Val  
 100 105 110

Val Val Arg Asn Phe Leu Lys Ala Lys Lys Thr Ser Arg Lys Leu Val  
 115 120 125

Glu Ile Met Met Ala Gly Ile Thr Lys Tyr Asp Leu Gly Ile Glu Ala  
 130 135 140

Asp His Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg  
 145 150 155 160

Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Tyr Met Arg  
 165 170 175

Tyr Lys Val Arg Gln Arg Glu Thr Leu Phe Ser Arg Phe Val Phe Ser  
 180 185 190

Asn His Pro Lys Leu Ile Asn Thr Pro Trp Tyr Tyr Asp Pro Ser Leu  
 195 200 205

Lys Leu Ser Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr  
 210 215 220

Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Gln Thr Val Thr Leu  
 225 230 235 240

Leu Ala Lys Asn Ile Val Ala Asn Lys Glu Cys Glu Phe Lys Arg Ile  
 245 250 255

Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr  
 260 265 270

Trp Leu Thr Met Leu Asp Lys Asp Lys Phe Leu Tyr Ser Pro Ile Ala  
 275 280 285

Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Ala  
 290 295 300

Glu Pro Gln Pro Val Glu Asn Gly Leu Pro Leu Glu Gly Leu Leu Gln  
 305 310 315 320

Ser Ile Ile Asn Lys Lys Pro Val Leu Ile Pro Ile Ala Gly Glu Gly  
 325 330 335

Ala Ser Gln Met Glu Ile Glu Arg Glu Thr His Phe Asp Gly Thr Asn

340							345					350				
Tyr	Leu	Ala	Ile	Arg	Pro	Gly	Val	Val	Ile	Gly	Tyr	Ser	Arg	Asn	Glu	
355							360					365				
Lys	Thr	Asn	Ala	Ala	Leu	Glu	Ala	Ala	Gly	Ile	Lys	Val	Leu	Pro	Phe	
370							375					380				
His	Gly	Asn	Gln	Leu	Ser	Leu	Gly	Met	Gly	Asn	Ala	Arg	Cys	Met	Ser	
385							390					395				
Met	Pro	Leu	Ser	Arg	Lys	Asp	Val	Lys	Trp							
405							410									

(2) INFORMATION FOR SEQ ID NO:8:

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 410 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Mycoplasma hominis*  
(B) STRAIN: PG21  
(G) CELL TYPE: unicellular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met	Ser	Val	Phe	Asp	Ser	Lys	Phe	Asn	Gly	Ile	His	Val	Tyr	Ser	Glu
1				5					10					15	
Ile	Gly	Glu	Leu	Glu	Thr	Val	Leu	Val	His	Glu	Pro	Gly	Arg	Glu	Ile
			20					25					30		
Asp	Tyr	Ile	Thr	Pro	Ala	Arg	Leu	Asp	Glu	Leu	Leu	Phe	Ser	Ala	Ile
		35					40					45			
Leu	Glu	Ser	His	Asp	Ala	Arg	Lys	Glu	His	Gln	Glu	Phe	Val	Lys	Ile
	50					55					60				
Met	Lys	Asp	Arg	Gly	Ile	Asn	Val	Val	Glu	Leu	Thr	Asp	Leu	Val	Ala
65					70					75					80
Glu	Thr	Tyr	Asp	Leu	Ala	Ser	Lys	Ala	Ala	Lys	Glu	Glu	Phe	Ile	Glu
				85					90					95	
Thr	Phe	Leu	Glu	Glu	Thr	Val	Pro	Val	Leu	Thr	Glu	Ala	Asn	Lys	Lys
			100					105					110		
Ala	Val	Arg	Ala	Phe	Leu	Leu	Ser	Gln	Lys	Pro	Thr	His	Glu	Met	Val
		115					120					125			



Glu Phe Met Met Ser Gly Ile Thr Lys Tyr Glu Leu Gly Val Glu Ser  
 130 135 140  
 Glu Asn Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg  
 145 150 155 160  
 Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Phe Met Arg  
 165 170 175  
 Tyr Ile Val Arg Arg Arg Glu Thr Leu Phe Ala Arg Phe Val Phe Arg  
 180 185 190  
 Asn His Pro Lys Leu Val Lys Thr Pro Trp Tyr Tyr Asp Pro Ala Met  
 195 200 205  
 Lys Met Pro Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Glu Thr  
 210 215 220  
 Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Asp Thr Ile Thr Leu  
 225 230 235 240  
 Leu Ala Lys Asn Ile Lys Ala Asn Lys Glu Val Glu Phe Lys Arg Ile  
 245 250 255  
 Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr  
 260 265 270  
 Trp Leu Thr Met Leu Asp Lys Asn Lys Phe Leu Tyr Ser Pro Ile Ala  
 275 280 285  
 Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Ala  
 290 295 300  
 Glu Pro Gln Pro Val Leu Asn Gly Leu Pro Leu Asp Lys Leu Leu Ala  
 305 310 315 320  
 Ser Ile Ile Asn Lys Glu Pro Val Leu Ile Pro Ile Gly Gly Ala Gly  
 325 330 335  
 Ala Thr Glu Met Glu Ile Ala Arg Glu Thr Asn Phe Asp Gly Thr Asn  
 340 345 350  
 Tyr Leu Ala Ile Lys Pro Gly Leu Val Ile Gly Tyr Asp Arg Asn Glu  
 355 360 365  
 Lys Thr Asn Ala Ala Leu Lys Ala Ala Gly Ile Thr Val Leu Pro Phe  
 370 375 380  
 His Gly Asn Gln Leu Ser Leu Gly Met Gly Asn Ala Arg Cys Met Ser  
 385 390 395 400  
 Met Pro Leu Ser Arg Lys Asp Val Lys Trp  
 405 410

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 410 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Mycoplasma orale
- (B) STRAIN: FERM BP-1970
- (G) CELL TYPE: unicellular

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

Met Ser Val Phe Ser Asp Lys Phe Asn Gly Ile His Val Tyr Ser Glu
 1             5             10             15

Ile Gly Asp Leu Glu Ser Val Leu Val His Glu Pro Gly Leu Glu Ile
 20             25             30

Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe Ser Ala Ile
 35             40             45

Leu Glu Ser Thr Asp Ala Arg Lys Glu His Lys Glu Phe Val Glu Glu
 50             55             60

Leu Lys Lys Gln Gly Ile Asn Val Val Glu Leu Val Asp Leu Val Val
 65             70             75             80

Glu Thr Tyr Asn Leu Val Asp Lys Lys Thr Gln Glu Lys Leu Leu Lys
 85             90             95

Asp Phe Leu Asp Asp Ser Glu Pro Val Leu Ser Pro Glu His Arg Lys
100            105            110

Ala Val Glu Lys Leu Leu Lys Ser Leu Lys Ser Thr Lys Glu Leu Ile
115            120            125

Gln Tyr Met Met Ala Gly Ile Thr Lys Tyr Asp Leu Gly Ile Lys Ala
130            135            140

Asp Lys Glu Leu Ile Val Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg
145            150            155            160

Asp Pro Phe Ala Ser Val Gly Asn Gly Val Thr Ile His Tyr Met Arg
165            170            175

Tyr Lys Val Arg Asn Arg Glu Thr Leu Phe Ser Lys Phe Ile Phe Thr
180            185            190

Asn His Pro Lys Leu Val Lys Thr Pro Trp Tyr Tyr Asp Pro Ala Met
195            200            205

```

Lys Leu Ser Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr  
 210 215 220  
 Leu Val Val Gly Val Ser Glu Arg Thr Asp Leu Glu Thr Ile Thr Leu  
 225 230 235 240  
 Leu Ala Lys Asn Ile Lys Ala Asn Lys Glu Cys Glu Phe Lys Arg Ile  
 245 250 255  
 Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu Asp Thr  
 260 265 270  
 Trp Leu Thr Met Leu Asp Lys Asp Lys Phe Leu Tyr Ser Pro Ile Ala  
 275 280 285  
 Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val Asn Gly Gly Ser  
 290 295 300  
 Asn Pro Glu Pro Val Val Asn Gly Leu Pro Leu Asp Lys Leu Leu Glu  
 305 310 315 320  
 Ser Ile Ile Asn Lys Lys Pro Val Leu Ile Pro Ile Ala Gly Lys Gly  
 325 330 335  
 Ala Thr Glu Ile Glu Thr Ala Val Glu Thr His Phe Asp Gly Thr Asn  
 340 345 350  
 Tyr Leu Ala Ile Lys Pro Gly Val Val Val Gly Tyr Ser Arg Asn Val  
 355 360 365  
 Lys Thr Asn Ala Ala Leu Glu Ala Asn Gly Ile Lys Val Leu Pro Phe  
 370 375 380  
 Lys Gly Asn Gln Leu Ser Leu Gly Met Gly Asn Ala Arg Cys Met Ser  
 385 390 395 400  
 Met Pro Leu Ser Arg Lys Asp Val Lys Trp  
 405 410

**WHAT IS CLAIMED IS:**

1. An isolated nucleic acid molecule encoding an arginine deiminase enzyme comprising an amino acid sequence of SEQ ID NO:2
2. The isolated nucleic acid molecule of claim 1, comprising a nucleic acid sequence of SEQ ID NO:1.
3. The isolated nucleic acid molecule of claim 2, wherein said nucleic acid sequence of SEQ ID NO:1 is mutated by nucleotide substitutions selected from the group consisting of a T at nucleotide 39, a C at nucleotide 104, an A at nucleotide 206, an A at nucleotide 729, a G at nucleotide 337, a C at nucleotide 830, a C at nucleotide 1023, an A at nucleotide 6, a T at nucleotide 15 and a C at nucleotide 18.
4. The isolated nucleic acid molecule of claim 1 that is selected from the group consisting of an RNA molecule and a DNA molecule.
5. An isolated nucleic acid molecule that is complementary to the nucleic acid molecule of claim 1.
6. An expression vector comprising the nucleic acid molecule of claim 1 operably linked to a control sequence.
7. The expression vector of claim 6 wherein said control sequence comprises a promoter.
8. The expression vector of claim 7 wherein said promoter is inducible.
9. The expression vector of claim 6, wherein said promoter is selected from the group consisting of a beta -lactamase promoter, a lac promoter, a trp promoter, a phoA promoter, an araBAD promoter, a T7 promoter, derivatives of the lambda PL and PR promotor and an O<sub>L</sub>/P<sub>R</sub> hybrid promoter.
10. The expression vector of claim 6, selected from the group consisting of a plasmid, a phage and a cosmid.
11. The clone designated as EN231 comprising the nucleic acid molecule of SEQ ID NO:1, containing plasmid pEN232.
12. A recombinant host cell comprising the nucleic acid molecule of claim 1.

13. The host cell of claim 12, selected from the group consisting of a prokaryotic host cell and a eukaryotic host cell.
14. The host cell of claim 12, selected from the group consisting of a bacterium and a yeast.
15. The host cell of claim 14, that is *Escherichia coli*.
16. An isolated arginine deiminase comprising the amino acid sequence of SEQ ID NO:2
17. The isolated arginine deiminase of claim 16, having a specific activity of from about 3 to about 30 IU/mg.
18. Arginine deiminase produced by a process comprising:
  - a) culturing the host cell of claim 12 and
  - b) expressing arginine deiminase in said host cell.
19. A process for producing arginine deiminase comprising:
  - a) culturing the host cell of claim 12 and
  - b) expressing arginine deiminase in said host cell.
20. A process for producing arginine deiminase comprising:
  - a) transforming or transfecting a host cell with the vector of claim 6;
  - b) culturing the transformed or transfected host cell; and
  - c) expressing arginine deiminase in said cultured host cell.
21. The process of claim 20, further comprising recovering said expressed arginine deiminase.
22. The process of claim 21, wherein said arginine deiminase is recovered by a process comprising extracting said expressed arginine deiminase from said host cell and renaturing said expressed arginine deiminase.
23. An isolated nucleic acid molecule that is amplified from a *Mycoplasma arthritidis* genome by a polymerase chain reaction using a primer pair selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:3 modified at base 6 so that A is substituted for T; at base 15 so that T is substituted for C; and at base 18 so that C is substituted for T, and combinations thereof.

24. A non-antigenic polymer-arginine deiminase conjugate, comprising purified arginine deiminase (ADI) obtained from *Mycoplasma arthritidis* covalently conjugated to a non-antigenic polymer.

25. The non-antigenic polymer-arginine deiminase conjugate of claim 24, wherein said ADI comprises amino acid SEQ ID NO:2.

26. The non-antigenic polymer-arginine deiminase conjugate of claim 24, wherein said non-antigenic polymer comprises a polyalkylene oxide.

27. The non-antigenic polymer-arginine deiminase conjugate of claim 26, wherein said polyalkylene oxide comprises an alkyl terminal.

28. The non-antigenic polymer-arginine deiminase conjugate of claim 26, wherein polyalkylene oxide is polyethylene glycol.

29. The non-antigenic polymer-arginine deiminase conjugate of claim 27, wherein said alkyl-terminated polyalkylene oxide is a monomethyl-terminated polyethylene glycol, (mPEG).

30. The non-antigenic polymer-arginine deiminase conjugate of claim 24, wherein said non-antigenic polymer has a molecular weight of from about 200 to about 60,000.

31. The non-antigenic polymer-arginine deiminase conjugate of claim 30, wherein said non-antigenic polymer has a molecular weight of from about 1,000 to about 40,000.

32. The non-antigenic polymer-arginine deiminase conjugate of claim 31, wherein said non-antigenic polymer has a molecular weight of from about 2,000 to about 12,500.

33. The non-antigenic polymer-arginine deiminase conjugate of claim 24, wherein said non-antigenic polymer is selected from the group consisting of dextran, polyvinyl pyrrolidones, polyacryl amides, polyvinyl alcohols and carbohydrate-based polymers.

34. The non-antigenic polymer-arginine deiminase conjugate of claim 24, further comprising a carbamate linkage between the arginine deiminase and non-antigenic polymer.

35. The non-antigenic polymer-arginine deiminase conjugate of claim 24, further comprising an amide linkage between the arginine deiminase and non-antigenic polymer.

36. A method of treating an arginine deiminase-susceptible condition in a mammal, comprising administering an effective amount of a composition comprising the

isolated arginine deiminase of claim 16 to a mammal.

37. A method of treating an arginine deiminase-susceptible condition in mammals, comprising administering an effective amount of the composition of claim 24.

38. A process for preparing a non-antigenic polymer-arginine deiminase conjugate, comprising contacting purified arginine deiminase (ADI) obtained from *Mycoplasmma arthritis* with a non-antigenic polymer under conditions sufficient to effect conjugation of said purified arginine deiminase and said non-antigenic polymer.

39. A product prepared according to the process of claim 21.

40. The method of claim 36, wherein said arginine deiminase-susceptible condition is an arginine deiminase susceptible tumor or cancer.

41. The method of claim 36, wherein said mammal is a human patient.

42. The method of claim 37, wherein said non-antigenic polymer comprises a polyalkylene oxide.

43. The method of claim 42, wherein said polyalkylene oxide comprises an alkyl terminal.

44. The method of claim 42, wherein said polyalkylene oxide is polyethylene glycol.

45. The method of claim 37, wherein said non-antigenic polymer has a molecular weight of from about 200 to about 35,000.

46. The method of claim 37, wherein said non-antigenic polymer has a molecular weight of from about 1,000 to about 15,000.

47. The method of claim 37, wherein said non-antigenic polymer has a molecular weight of from about 2,000 to about 12,500.

48. The method of claim 37, wherein said non-antigenic polymer is selected from the group consisting of dextran, polyvinyl pyrrolidones, polyacryl amides, polyvinyl alcohols and carbohydrate-based polymers.

49. The method of claim 37, said conjugate further comprising a carbamate linkage between the arginine deiminase and non-antigenic polymer.

50. The method of claim 37, said conjugate further comprising an amide linkage between the arginine deiminase and non-antigenic polymer.

# FIG-1 ADI SEQ ID:1

```

ATG TCT GTA TTT GAC AGT AAA TTT AAG GGA ATT CAT GTC TAT TCA   45
GAA ATT GGT GAA CTA GAA ACC GTT TTA GTT CAC GAA CCT GGT AAA   90
GAA ATT GAT TAC ATT ACC CCA GCT CGT TTG GAT GAA TTA TTA TTC  135
TCA GCT ATT CTA GAA AGC CAC GAT GCA AGA AAA GAA CAC AAA GAA  180
TTC GTA GCA GAA CTT AAA AAG CGT GGA ATT AAT GTT GTT GAA TTA  225
GTA GAT CTA ATC GTA GAA ACC TAT GAT TTA GCA TCA AAA GAA GCT  270
AAA GAA AAA CTT TTA GAA GAA TTC CTA GAT GAT TCA GTA CCA GTT  315
CTA TCA GAC GAA CAC CGT GCT ACT GTT AAG AAA TTC TTA CAA AGT  360
CAA AAA TCA ACA AGA TCA TTA GTT GAA TAC ATG ATC GCA GGG ATC  405
ACT AAA CAC GAT TTA AAA ATC GAA TCA GAT TTA GAA TTA ATC GTT  450
GAC CCA ATG CCT AAC TTG TAC TTC ACT CGT GAC CCA TTT GCA TCA  495
GTA GGT AAT GGA GTT ACC ATC CAC TAC ATG CGT TAC AAA GTA AGA  540
CAA CGT GAA ACA TTA TTT AGC CGA TTT GTA TTT TCA AAT CAC CCT  585
AAA CTA GTT AAT ACC CCA TGG TAC TAC GAC CCT GCT GAA GGA TTA  630
ACA ATC GAA GGT GGA GAC GTA TTT ATC TAC AAT AAC GAT ACT TTA  675
GTA GTT GGT GTT TCA GAA AGA ACT GAC TTA CAA ACT ATT ACT TTA  720
TTA GCT AAG AAC ATT AAA GCA AAT AAA GAA TGT GAA TTC AAA CGT  765
ATT GTA GCA ATT AAT GTT CCT AAA TGG ACA AAC CTA ATG CAC TTA  810
GAC ACA TGG TTA ACA ATG CTA GAC AAA GAT AAA TTC TTA TAC TCA  855
CCT ATT GCA AAT GAT GTG TTT AAA TTC TGG GAC TAC GAT TTA GTT  900
AAT GGC GGA GAC GCT CCT CAA CCA GTT GAC AAT GGA TTA CCT CTA  945
GAA GAC TTA TTG AAA TCA ATC ATT GGT AAG AAA CCT ACT CTA ATT  990
CCT ATT GCT GGT GCT GGT GCT TCA CAA ATC GAT ATT GAA CGT GAA 1035
ACC CAC TTT GAC GGA ACA AAC TAC CTA GCT GTA GCT CCT GGA ATT 1080
GTT ATT GGT TAT GCA CGT AAC GAA AAA ACA AAT GCC GCT TTA GAA 1125
GCT GCA GGA ATT ACT GTT CTA CCA TTC AGA GGA AAC CAA CTT TCA 1170
CTT GGA ATG GGA AAT GCT CGT TGC ATG TCA ATG CCT CTA TCA CGT 1215
AAA GAT GTT AAG TGG                                     1230

```



# FIG-2 SEQ ID NO:2

```

Met Ser Val Phe Asp Ser Lys Phe Lys Gly Ile His Val Tyr Ser   15
Glu Ile Gly Glu Leu Glu Thr Val Leu Val His Glu Pro Gly Lys   30
Glu Ile Asp Tyr Ile Thr Pro Ala Arg Leu Asp Glu Leu Leu Phe   45
Ser Ala Ile Leu Glu Ser His Asp Ala Arg Lys Glu His Lys Glu   60
Phe Val Ala Glu Leu Lys Lys Arg Gly Ile Asn Val Val Glu Leu   75
Val Asp Leu Ile Val Glu Thr Tyr Asp Leu Ala Ser Lys Glu Ala   90
Lys Glu Lys Leu Leu Glu Glu Phe Leu Asp Asp Ser Val Pro Val  105
Leu Ser Asp Glu His Arg Ala Thr Val Lys Lys Phe Leu Gln Ser  120
Gln Lys Ser Thr Arg Ser Leu Val Glu Tyr Met Ile Ala Gly Ile  135
Thr Lys His Asp Leu Lys Ile Glu Ser Asp Leu Glu Leu Ile Val  150
Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg Asp Pro Phe Ala Ser  165
Val Gly Asn Gly Val Thr Ile His Tyr Met Arg Tyr Lys Val Arg  180
Gln Arg Glu Thr Leu Phe Ser Arg Phe Val Phe Ser Asn His Pro  195
Lys Leu Val Asn Thr Pro Trp Tyr Tyr Asp Pro Ala Glu Gly Leu  210
Thr Ile Glu Gly Gly Asp Val Phe Ile Tyr Asn Asn Asp Thr Leu  225
Val Val Gly Val Ser Glu Arg Thr Asp Leu Gln Thr Ile Thr Leu  240
Leu Ala Lys Asn Ile Lys Ala Asn Lys Glu Cys Glu Phe Lys Arg  255
Ile Val Ala Ile Asn Val Pro Lys Trp Thr Asn Leu Met His Leu  270
Asp Thr Trp Leu Thr Met Leu Asp Lys Asp Lys Phe Leu Tyr Ser  285
Pro Ile Ala Asn Asp Val Phe Lys Phe Trp Asp Tyr Asp Leu Val  300
Asn Gly Gly Asp Ala Pro Gln Pro Val Asp Asn Gly Leu Pro Leu  315
Glu Asp Leu Leu Lys Ser Ile Ile Gly Lys Lys Pro Thr Leu Ile  330
Pro Ile Ala Gly Ala Gly Ala Ser Gln Ile Asp Ile Glu Arg Glu  345
Thr His Phe Asp Gly Thr Asn Tyr Leu Ala Val Ala Pro Gly Ile  360
Val Ile Gly Tyr Ala Arg Asn Glu Lys Thr Asn Ala Ala Leu Glu  375
Ala Ala Gly Ile Thr Val Leu Pro Phe Arg Gly Asn Gln Leu Ser  390
Leu Gly Met Gly Asn Ala Arg Cys Met Ser Met Pro Leu Ser Arg  405
Lys Asp Val Lys Trp                                         410

```

## FIG-3

a MSVFDSKFKGIHVYSEIGELETVLVHEPGKEIDYITPARLDELLFSAILE 50  
 b -----S-----R-----  
 c -----N-----R-----  
 d ---SD--N-----D--S-----L-----

a SHDARKEHKEFVAELKKRGINVVELVDLIVETYDLASKEAKEKLLEFLD 100  
 b -----Q-----AND-----I--VA-----Q---D--I---E  
 c -----QS--KIM-D-----T--VA-----A---EFI-T--E  
 d -T-----EI--Q-----V---N-VD-KTQ---KD---

a DSVPVLSDEHRATVKKFLQSQKSTRSLVEYMIAGITKHDLKIESDLELIV 150  
 b --E----E--KVV-RN--KAK-TS-K---I-M-----Y--G--A-H-----  
 c ET----TEANKKA-RA--L-. -P-HEM--F-MS----YE-GV--EN----  
 d --E----P---KA-E-L-K-L---KE-IQ--M-----Y--G-KA-K---

a DPMPNLYFTRDPFASVGNGVTIHYMRYKVRQRETLFSRFVFSNHPKLVNT 200  
 b -----I---  
 c -----F--I--R---A---R-----K-  
 d -----N---K-I-T-----K-

a PWYYDPAEGLTIEGGDVFIYNNDTLVVGVSERTDLQTITLLAKNIKANKE 250  
 b -----SLK-S-----V-----V---  
 c -----MKMP-----E-----D-----  
 d -----MK-S-----E-----

a CEFKRIVAINVPKWTNLMHLDTWLTMLDKDKFLYSPIANDVFKFWDYDLV 300  
 b -----  
 c V-----N-----  
 d -----

a NGGDAPQPVDNGLPLEDLLKSIIGKKPTLIPIAGAGASQIDIERETHFDG 350  
 b ---AE----E-----G--Q---N--V-----E---ME-----  
 c ---AE---QL-----DK--A--N-E-V---G---TEME-A--N---  
 d ---SN-E--V-----DK--E--N--V-----K--TE-ETAV-----

a TNYLAVAPGIVIGYARNEKTNAALEAAGITVLPFRGNQLSLGMGNARCMS 400  
 b -----IR--V---S-----K---H-----  
 c -----IK--L--D-----K-----H-----  
 d -----IK--V-V--S--V-----N--K---K-----

a MPLSRKDVKW 410  
 b -----  
 c -----  
 d -----